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Variability in Free 25(OH) Vitamin D Levels in Clinical Populations

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Abstract

Our goal was to determine total and directly measured free 25-hydroxy vitamin D (25(OH)D) serum levels in humans with a range of 25(OH)D levels and clinical conditions associated with low and high vitamin D binding protein levels. Serum samples and clinical data were collected from 106 subjects: 62 without cirrhosis or pregnancy, 24 cirrhotic patients with albumin <2.9 g/dL, and 20 pregnant women. Total 25(OH)D (LC/MS/MS) and “free” 25(OH)D (immunoassay) were measured. Total 25(OH)D was significantly lower in liver disease patients but free 25(OH)D concentrations were significantly higher in this group ($p < .001$). Neither total nor free 25(OH)D concentrations were significantly different in pregnant women vs. the comparator group. There were significant direct positive relationships between free 25(OH)D and total 25(OH)D concentrations for the entire dataset and for each group ($p < .0001$), however slopes of relationships differed in the cirrhotic group compared to pregnant women or the comparator group. In cirrhotics: y (free 25(OH)D) = $2.52 + 0.29 * X$ (total 25(OH)D), $r^2 = .51$, $p < .001$; $y = 1.45 + 0.09 * X$; $r^2 = .77$, $p < .0001$ for pregnant women; and $y = 1.11 + 0.12 * X$; $r^2 = .72$, $p < .0001$ for the comparator group). Conclusions: directly measured free 25(OH)D serum concentrations and relationships between total and free 25(OH)D vary with clinical conditions, and may differ from those predicted by indirect estimation methods.

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Keywords

vitamin D; 25(OH)vitamin D; free 25(OH) vitamin D; cirrhosis; pregnancy

INTRODUCTION

There continues to be debate about assessment of vitamin D status in humans based on circulating vitamin D or metabolite concentrations. There is agreement, however, that adequate status should be defined by concentrations of serum 25-hydroxyl vitamin D (25(OH)D) (1–3) as this metabolite reflects overall body storage of the immediate vitamin D precursor that is hydroxylated to active 1,25-dihydroxy(OH)₂ vitamin D. Both circulating 1, 25(OH)₂ D and 25(OH)D bind to albumin and D binding protein leaving only a small fraction unbound or “free”. Receptor and drug theory posit that only the “free” compound is available to bind to receptor. Or in the case of 25(OH)D, only free is available for conversion to active 1, 25(OH)₂ D and thus, may more closely reflect the biologic activity. The potential benefit of measuring “free or unbound” concentrations of D and its metabolites, especially in the presence of biologic conditions that alter levels of the carrier proteins, has been suggested. (4–7). Until recently, determination of serum free 25(OH)D was an arduous undertaking involving some form of equilibrium dialysis or indirect estimation based on measurement of D binding protein, albumin, and 25(OH)D (using D standards and assays that were variable) with equations derived from relatively small numbers of people (4) or modified from equations used for sex hormones (8) . An assay that directly measures serum free 25(OH)D levels has been developed (Future Diagnostics B.V., Wijchen, The Netherlands). The purpose of this investigation was to determine total and directly measured free 25(OH)D in humans with a range of 25(OH)D levels and clinical conditions associated with a range of D binding proteins levels.

MATERIALS AND METHODS

Subjects

Stable subjects with liver disease and evidence of protein synthesis dysfunction defined as an albumin concentration of <2.9g/dL, women in their second and third trimester of pregnancy, and medically stable community-dwelling adults without evidence of liver disease or pregnancy provided informed consent and venous blood samples as part of protocols approved by the University of California, San Francisco Committee on Human Research. Samples were collected during October through December . Sunshine exposure was assessed using a questionnaire (9)

Laboratory Measurements

Total 25(OH)D measurements were determined by CLIA certified liquid chromatography tandem mass spectrometry at Mayo Clinical Laboratories with participation in National Institutes of Health Office of Dietary Supplements funded National Institute of Standards and Technology (NIST) quality assurance program for analysis of D metabolites in human serum. The assay has ~10% CV at levels 10 ng/mL. Internal standard is NIST reference standard. Free 25(OH)D concentrations were determined by immunoassay (Future

Diagnostics B.V., Wijchen, The Netherlands, <http://www.future-diagnostics.nl/>) In this assay an anti-vitamin D antibody is coated on a microtiterplate. Free 25(OH)D is captured by the antibody during a first incubation. After washing, a biotin-labeled 25(OH)D analog is allowed to react with the non-occupied antibody binding sites in a second incubation. After washing and incubation with a streptavidin-peroxidase conjugate, bound enzyme is quantitated using a colorimetric reaction. Intensity of the signal is inversely proportional to the level of free 25(OH)D in the sample. The assay was calibrated against a symmetric dialysis method.

Statistical Design and Data Analysis

Demographic and baseline characteristics of groups are presented as mean $\pm$ S. D. and compared using ANOVA for continuous variables and Chi square for bivariate measures. Relationships between total and free 25(OH)D concentrations were tested by linear regression and between group differences by ANOVA.

RESULTS

One hundred and six subjects participated. Characteristics by group (cirrhotic, pregnant, and comparator) and mean values for total and free 25(OH)D are presented in Table 1. Cirrhotic patients had a mean Model for End-Stage Liver Disease MELD score of 16 ± 3 with Child Pugh score (10, 11) B in eight and C in twelve. Half of the pregnant women were in the second trimester of pregnancy and half were in the third. The comparator group had a mean Charlson co-morbidity score (12) of 3.7 ± 3.4 . In the comparator group, five women were taking oral contraceptives and two received post-menopausal hormone replacement; no other sex hormones were taken. Supplemental vitamin consumption was frequent in the pregnant women (18 of 20) with prenatal vitamins containing 400 IU/day D. One third of the cirrhotics also reported daily multivitamin with mineral use with 400 IU vitamin D and 3 reported daily vitamin D supplementation (1000 IU/day). Only one person in the comparator group reported consumption of a multivitamin with 4000 IU D ($p < .0001$). Reported sunshine exposure was not significantly different between the groups, although variations were high (mean of 1.9 ± 1.8 hr/day in the summer in the cirrhotics, 3.0 ± 2.8 hr in pregnant women and 2.7 ± 2.2 in the comparator group, $p = 0.3$) and 1.6 ± 2.1 hr/day in cirrhotics, 1.9 ± 1.9 in pregnant women, and 1.9 ± 1.5 hr/day in the winter months in the comparator group, $p = 0.7$). Less than 10 per cent in any group used sunscreen. Dietary intake was not analyzed.

Total 25(OH)D was significantly lower in cirrhotic patients with protein synthesis dysfunction but free 25(OH)D concentrations were significantly higher in this group. Neither total nor free 25(OH)D concentrations were significantly different in pregnant women compared to the comparator group. There were significant direct positive relationships between free 25(OH)D and total 25(OH)D concentrations for the entire dataset and for each group ($p < .001$), however the slopes of the relationships differed in the cirrhotic group compared to the pregnant women or the comparator group (free 25(OH)D) $y = 2.5 + 0.29 * X$ (total 25(OH)D), $r^2 = 0.51$, $p < .001$ cirrhotics; $y = 1.5 + 0.09 * X$; $r^2 = 0.77$, $p < .0001$ for pregnant women; and $y = 1.11 + .12 * X$; $r^2 = 0.72$, $p < .0001$ for the comparator group).

DISCUSSION

Variation in D binding protein levels and binding properties affect the free fraction of circulating 25(OH)D. (4, 5, 13–15) Diseases such as cirrhosis that result in decreased protein synthetic capacity result in lower D binding protein concentrations and this would be anticipated to increase free 25(OH)D concentrations. Conversely, the state of pregnancy, especially during the second and third trimesters when D binding protein concentrations increase, would be expected to result in decreased free 25(OH)D concentrations. Indirect methods of calculating free D are highly dependent on D binding protein measurements and estimations will change in relation to changes in D binding protein. (4, 5, 13–15) As expected, we found patients with cirrhosis and low albumin concentrations had free 25(OH)D levels that were higher for any given total 25(OH)D level than in the pregnant women or comparator group. Surprisingly not only was the ratio of free to total 25(OH)D higher but the actual measured level of free 25(OH)D was higher in patients with cirrhosis. Also surprising was that women in their second and third trimester of pregnancy did not have lower free 25(OH) D concentrations than the comparator group despite predicted increased DBP levels. However, these results are consistent with the observation made by Bikle et al. (16) that the affinity of DBP for the vitamin D metabolites appears to be decreased during pregnancy perhaps to compensate for the increased DBP concentrations without decreasing the free metabolite levels.

Sunshine exposure has been shown to affect total 25(OH)D levels resulting in seasonal variation as well as geographic variation (17–20). We collected samples during seasons associated with lower sunshine exposure (fall, winter) and did not find differences in reported sunshine exposure between groups to explain differences in total 25(OH)D levels. We are not aware of reports of effects of season on concentrations of free 25(OH)D levels although they might be expected to parallel those of total 25(OH)D. In addition to season, vitamin D intake can affect 25(OH)D levels. Almost all the pregnant women and one third of the cirrhotic patients took multivitamins with vitamin D content of about 400 IU/day while the comparator group did not use supplements. This makes the lower concentrations in the cirrhotics even more striking and may also explain the lack of differences in total 25(OH)D between pregnant women and the comparator group. The effects and relationships between D intake and free 25(OH) levels have not been described to date.

CONCLUSIONS

Relationships between total and free 25(OH)D vary with clinical conditions that affect circulating protein concentrations, and may differ from predictions based on physiologic changes in circulating vitamin D binding protein and albumin. Direct measurement of free 25(OH) D warrants further evaluation to determine its clinical relevance in defining optimal vitamin D status for differing clinical conditions.

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Key Points

- Directly measured free 25-OH D was higher in patients with cirrhosis and low albumin despite lower total 25-OH D levels
- Relationships between free and total 25-OH D were also altered in cirrhotics
- Free 25-OH \ D levels in pregnant women did not differ from non-pregnant, non-cirrhotic people
- Free 25-OH D levels vary in clinical conditions and in ways that might not be predicted

Figure 1.

Total 25(OH) D concentrations are plotted on the x axis and directly measured free 25(OH)D levels on the y axis. Closed circles represent data from patients with cirrhosis, triangles represent data from pregnant women, and open circles represent data from the comparison group with the dashed line representing regression fits for the comparator group and solid lines presenting regressions for the cirrhotics and pregnant women.. Free 25(OH)D concentrations were correlated with total 25(OH)D concentrations in all groups, but cirrhotics had higher free 25(OH) D concentrations despite lower total 25(OH)D concentrations. Relationships between free 25-OH D and total 25-OH D in pregnant women did not differ from the comparator group.

Table 1

Results by Group

	<u>Cirrhotics</u>	<u>Pregnant</u>	<u>Comparison Group</u>
Number (n)	24	20	62
Women / Men (n)	15/9	20/0	31/31
Age (y)	57 (8) [*]	30 (7)	55 (17)
Race (Cauc/AfrAmer/Asian/Other)	16/2/2/4	15/4/1/0	42/14/5/1
Body Mass Index (M ²)	32.1 (7.4)	31 (5.2)	29.2 (7.0)
Albumin (g/dL) [#]	2.4 (0.4)	3.6 (0.3)	n.a.
Calcium (mg/dL) [#]	8.4 (0.5)	9.1 (0.6)	9.4 (0.4)
Total 25(OH)D (ng/mL) [#]	14 (7) ^{**}	27 (10)	22 (13)
Free 25(OH)D (pg/mL) [#]	6.6 (3.0) ^{**}	4 (1.1)	3.8 (1.9) [^]
Per Cent Free	0.05 (.02) ^{**}	0.02 (.00)	0.02 (.01) [^]

^{*} Data are mean (S.D.)

^{**} p<.001 for group differences for liver disease vs. other groups.

[^] ns for differences between pregnant women and women in comparator group.

[#] for conversion to S.I. units: albumin- multiple by 10 for g/L; calcium multiply by 0.25 for mmol/L; for 25(OH)D multiply by 2.496 (for nmol/L or pmol/L)